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Involvement of Peptidylprolyl *cis*/*trans* Isomerases in *Enterococcus faecalis* Virulence

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Peptidylprolyl *cis*/*trans* isomerases (PPIases) are enzymes involved in protein folding. Analysis of the genome sequence of *Enterococcus faecalis* V583 allowed for identification of 3 PPIases carrying genes. *ef2898* encodes an intracellular PPIase which was not shown to be important for the *E. faecalis* stress response or virulence. The other two PPIases, the parvulin family rotamase EF0685 and the cyclophilin family member EF1534, are expected to be surface-exposed proteins. They were shown to be important for virulence and resistance to NaCl. A Δ*ef0685* Δ*ef1534* mutant was also more resistant to oxidative stress, was able to grow under a high manganese concentration, and showed altered resistance to ampicillin and quinolone antibiotics.

Peptidylprolyl *cis*/*trans* isomerases (PPIases; also named foldases or maturases) are ubiquitous folding proteins (present in eukaryote or prokaryote cells) catalyzing the *cis*/*trans* isomerization of peptidylprolyl. They can be intra- or extracellular and are divided into three typical foldase families, including the cyclophilins, which are able to bind the immunosuppressant cyclosporine, the FK506 drug-binding proteins (FKBP), and the parvulin family, which refers to the described *Escherichia coli* parvulin (13, 29). These foldases ensure the conformation of proteins involved in many vital functions but also in virulence. In Gram-negative bacteria, SurA is a parvulin periplasmic folding factor involved in maturation of outer membrane proteins. SurA is also important for adhesion and invasion of *E. coli*, *Shigella flexneri*, and *Salmonella enterica* serovar Typhimurium (4). This protein is also involved in the oxidative stress resistance and virulence of *Yersinia pseudotuberculosis* (27). The macrophage infectivity potentiator (MIP) is a FKBP foldase necessary for the adhesion to collagen, and its PPIase activity renders the extracellular matrix sensitive to serine protease, which allows the invasion of *Legionella pneumophila* through the epithelial barrier (15). In Gram-positive bacteria, PPIases were also involved in bacterial virulence. For example, the maturase lipoprotein of the parvulin family PrtM was shown to be important for *Streptococcus equi* colonization in mouse and pony models (12). The parvulin foldase PrsA is essential for *Bacillus subtilis* viability (16) and required for protein secretion and production of fully mature streptococcal pyogenic exotoxin B (SpeB) in group A *Streptococcus* (22). In *Listeria monocytogenes*, the lipoprotein chaperone PrsA2 is involved in virulence factor secretion and viability within host cell cytosol and is regulated by the regulator of virulence factors PrfA (1). Among the four PPIases identified in *Streptococcus pneumoniae*, two are lipoproteins and, consequently, surface located. One of them, the rotamase lipoprotein SlrA (streptococcal rotamase A), belongs to the cyclophilin family, while the second one, PpmA (proteinase maturation protein A), shares homologies to PPIases of the parvulin family. Both are involved in the colonization phase of infection and in the evasion of phagocytes. The construction of an *slrA ppmA* double mutant showed a phenotype of reduced virulence slightly more important than those of the corresponding single mutants (7, 13, 28). These examples highlight the importance of PPIases from Gram-positive bacteria in virulence.

Enterococcus faecalis is an opportunistic bacterium normally present in the human gastrointestinal tract which became an emergent pathogen responsible for nosocomial infections in many hospitals in the United States and Europe (26). Involvement of 3 *E. faecalis* PPIases in stress response or in virulence was investigated here. Among them, 2 of these (EF0685 and EF1534) are lipoproteins anchored to the membrane and are predisposed to be involved in the folding of secreted proteins.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. faecalis* V19 parental strain (Table 1) used in this study is a plasmid-cured derivative of the vancomycin-resistant clinical isolate V583 (38). *E. faecalis* V19 and its derivates were cultivated at 37°C in M17 medium (36) supplemented with 0.5% glucose (GM17) or in M17 MOPS (morpholinepropanesulfonic acid) medium supplemented with glycerol as the sole carbohydrate (17). For growth under a high concentration of manganese, a chemically defined medium, MCDE Caa Trp medium (32), was used. *E. coli* XL1-Blue (Table 1) was used as the recipient for cloning. *E. coli* strains were cultivated under conditions of vigorous agitation at 37°C in Luria Bertani medium (33). When required, ampicillin (100 μg/ml) or erythromycin (150 μg/ml) was added to bacterial cultures.

General molecular methods. Genomic DNA extraction was performed as described by Sambrook et al. (33). GoTaq Flexi DNA polymerase (Promega, Madison, WI) was used for PCRs with the primers listed in Table 2. PCR products were purified using the NucleoSpin extract II kit (Macherey-Nagel, Dünen, Germany). Plasmids used in this study are listed in Table 1. They were extracted with the NucleoSpin plasmid kit (Macherey-Nagel). Restriction endonucleases and T4 DNA ligase were

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant property(ies)	Reference or source
E. faecalis strains		
V19	Plasmid-cured strain obtained from *E. faecalis* V583	38
Δ*ef0685*	*ef0685* deletion mutant obtained from V19	This study
Δ*ef0685C*	Δ*ef0685* mutant derivative complemented for *ef0685*	This study
Δ*ef1534*	*ef1534* deletion mutant obtained from V19	This study
Δ*ef1534C*	Δ*ef1534* mutant derivative complemented for *ef1534*	This study
Δ*ef0685* Δ*ef1534*	Mutant with deletions in *ef0685* and *ef1534* obtained from V19	This study
Δ*ef1534* Δ*ef2898*	Mutant with deletions in *ef1534* and *ef2898* obtained from V19	This study
Δ*ef1534C* Δ*ef2898*	Δ*ef1534* Δ*ef2898* derivative complemented for *ef1534*	This study
Δ*ef1534* Δ*ef2898C*	Δ*ef1534* Δ*ef2898* derivative complemented for *ef2898*	This study
E. coli strain XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F′ *proAB lacI*^{q}*Z*ΔM15] Tn10 (Tet^r)	Stratagene
Plasmids		
pGEMt	System for cloning PCR products	Promega
pGEMtwt*ef0685*	pGEMt derivative carrying a 4.0-kb DNA fragment from *ef0685*	This study
pGEMtwt*ef1534*	pGEMt derivative carrying a 2.9-kb DNA fragment from *ef1534*	This study
pGEMtwt*ef2898*	pGEMt derivative carrying a 3.3-kb DNA fragment from *ef2898*	This study
pGHost9	Plasmid with thermosensitive replication	24
pGHost9wt*ef0685*	pGHost9 derivative carrying a 4.0-kb DNA fragment from *ef0685*	This study
pGHost9wt*ef1534*	pGHost9 derivative carrying a 2.9-kb DNA fragment from *ef1534*	This study
pGHost9wt*ef2898*	pGHost9 derivative carrying a 3.3-kb DNA fragment from *ef2898*	This study
pGHost9Δ*ef0685*	pGHost9 derivative carrying a 3.1-kb DNA fragment with a deleted *ef0685* gene	This study
pGHost9Δ*ef1534*	pGHost9 derivative carrying a 2.3-kb DNA fragment with a deleted *ef1534* gene	This study
pGHost9Δ*ef2898*	pGHost9 derivative carrying a 2.8-kb DNA fragment with a deleted *ef2898* gene	This study

purchased from Promega and used according to the manufacturer's instructions.

Construction of mutants affected in *ef0685*, *ef1534*, and *ef2898* and complementation. For the construction of an *ef0685* deletion mutant (Δ*ef0685*), allelic replacement was used as described below. Ninety percent of the *ef0685* gene was deleted in the mutant strain, starting 27 bp after the ATG start codon and stopping 65 bp before the TAA stop codon. Briefly, a 4.0-kb DNA fragment (obtained using chromosomal DNA of *E. faecalis* V19 as the template) containing the *ef0685* gene flanked by 1,486 bp upstream and 1,456 bp downstream was amplified using primers Ef0685m1 and Ef0685m4 (Table 2) and cloned into the pGEMt plasmid (Table 1) to obtain pGEMtwt*ef0685*. Then, an inverse PCR was done with primers Ef0685m2 and Ef0685m3 (Table 2) to allow a 930-bp deletion. After digestion with BamHI, the fragment was recircularized with T_4 DNA ligase. The common SacII restriction site between the pGEMt and pGHost9 plasmids (Table 1) was used to add pGHost9 to the previous construction. The next step was a digestion with the restriction endonuclease NotI to remove the pGEMt vector and to obtain a plasmid composed of pGHost9 with the deleted fragment of *ef0685* (pGHost9Δ*ef0685*). The corresponding recombinant plasmid was then introduced in *E. faecalis* V19. Transformants obtained at 30°C (erythromycin-resistant colonies) were used for temperature shifts in order to select clones with the pGHost9Δ*ef0685* plasmid integrated within the chromosome (first crossing-over) and then clones resulting from a double crossing-over. These were analyzed for the presence of a deleted *ef0685* gene, and the corresponding region was verified by sequencing.

TABLE 2 Primers used in this study

Primer name	Sequence (5′–3′)[a]
Ef0685m0	GCAGGAAACCATTCCAATTT
Ef0685m1	TACGAGCTCGTTTGAACGCTTCTT (SacI)
Ef0685m2	ACTGGATCCGCTGCAGCTAAGATT (BamHI)
Ef0685m3	TTCGGATCCCAAAACAAGCGACTC (BamHI)
Ef0685m4	TCTGCATGCACTAGCTCAACGTTGGTCT (SphI)
Ef0685m5	ACCTTAGATGAGTTGTATCAA
Ef1534m0	TGAGACAATTTTGTTGCGAC
Ef1534m1	CACGAATTCTACCTCTTGCGCTATGCTCC (EcoRI)
Ef1534m2	TCAGGATCCGTTCCCCTCGTGCCTTTA (BamHI)
Ef1534m3	CAAGGATCCAAAGTCGAAGTTGGTGCC (BamHI)
Ef1534m4	AATACTGCAGATGTCGGTGCCTCAG (PstI)
Ef1534m5	AAATCGTTATTTCATCGTATGC
Ef2898m0	GATGATAAATGATCGGTAGATG
Ef2898m1	TTCTGTCGACCCACAGCTTTCACTTGTTCC (SalI)
Ef2898m2	TCAGGATCCGCTTTTGGGCCTTTAGC (BamHI)
Ef2898m3	CAAGGATCCGTCCAACATTTGACGTCG (BamHI)
Ef2898m4	AATTCCATGGCGTATACAGCACCTGCTG (NcoI)
Ef2898m5	GAAATTTTGTTTAATAGTGGCG
PU	TGTAAAACGACGGCCAGT
PR	CAGGAAACAGCTATGACC

[a] The underlined sequences correspond to the recognition sites of the restriction endonucleases shown in parentheses.

For the construction of the *ef1534* and *ef2898* deletion mutants (Δ*ef1534* and Δ*ef2898*, respectively), the same strategy was used. Primers Ef1534m1 and Ef1534m4 for *ef1534* and Ef2898m1 and Ef2898m4 for *ef2898* (Table 2) allowed for amplification of 2.9-kb and 3.3-kb DNA fragments, respectively, which were cloned in pGEMt to produce pGEMtwt*ef1534* and pGEMtwt*ef2898* (Table 1). Inverse PCRs were performed with oligonucleotides Ef1534m2/Ef1534m3 and Ef2898m2/Ef2898m3 to generate 565-bp and 467-bp deletions for *ef1534* and *ef2898*, respectively. With the method described above, pGHost9 was added to these constructions, and then pGEMt was removed to obtain pGHost9Δ*ef1534* and pGHost9Δ*ef2898*. The corresponding recombinant plasmids were introduced in *E. faecalis* V19, and temperature shifts allowed mutants to be obtained. These were analyzed for the presence of a deleted gene, and the corresponding region was verified by sequencing. We constructed the Δ*ef0685* Δ*ef1534* and Δ*ef1534* Δ*ef2898* double mutants using the Δ*ef0685* strain and pGHost9Δ*ef1534* and Δ*ef1534* and pGHost9Δ*ef2898*, respectively.

To complement the *ef0685* mutation, the 4.0-kb PCR fragment con-

taining the wild-type (wt) *ef0685* gene (Ef0685m1-Ef0685m4) was inserted into pGHost9 to obtain pGHost9wt*ef0685*. This plasmid was then introduced into the Δ*ef0685* mutant, and double crossover events allowed the construction of the complemented Δ*ef0685*C strain. The same strategy was used to complement the Δ*ef1534* and Δ*ef2898* mutants with plasmids pGHost9wt*ef1534* and pGHost9wt*ef2898*, respectively. All complemented strains were analyzed by PCR, and the corresponding region was verified by sequencing.

Survival experiments. *E. faecalis* survival to sodium chloride (28.5%), bile salts (0.3%), ethanol (22%), heat (62°C), and incubation in urine from humans (healthy volunteers) was tested as described by Reffuveille et al. (32). Susceptibility of *E. faecalis* strains to antibiotics was estimated using Mueller-Hinton medium plates with discs containing ampicillin (10 μg), penicillin (6 μg), gentamicin (500 μg), kanamycin (1 mg), netilmicin (30 μg), streptomycin (500 μg), tobramycin (10 μg), doxycycline (30 μg), minocycline (30 IU), tetracycline (30 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), nalidixic acid (30 μg), norfloxacin (5 μg), ofloxacin (5 μg), teicoplanin (30 μg), or vancomycin (30 μg).

Detection of SOD activity. Bacteria from exponential growth phase in GM17 medium (optical density at 600 nm [OD_{600}] of 0.5) were exposed to 1.5 mM H_2O_2 for 30 min. Total proteins were then extracted, and 15 μg was separated by nondenaturing PAGE (carried out according to the protocol of Laemmli [18], except that SDS and β-mercaptoethanol were omitted). The superoxide dismutase (SOD) activity was detected using the protocol of Beauchamp and Fridovich (3).

Infection and survival experiments of *Galleria mellonella*. Infection of *G. mellonella* larvae with *E. faecalis* was accomplished as previously described by Lebreton et al. (19). Briefly, using a syringe pump (KD Scientific, Holliston, MA), larvae (about 0.3 g and 3 cm in length) were infected subcutaneously with washed *E. faecalis* cells from an overnight culture in GM17, with $1.5 \times 10^6 \pm 0.15 \times 10^6$ or $3 \times 10^6 \pm 0.3 \times 10^6$ CFU per larva administered in 10 μl of sterile saline buffer. In each test, 15 insects were infected, and the experiments were repeated at least three times. Larval killing was then monitored each hour from 18 h to 24 h postinfection.

Caco-2/TC7 culture and infection. Caco-2/TC7 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 15% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml each of penicillin and streptomycin, and 1% nonessential amino acids. For experimental assays, the cells were seeded at a density of approximately 1×10^5 cells/cm^2 in 24-wells tissue culture plates cultured at 37°C in 5% CO_2-95% air atmosphere, and the medium was changed daily. For infection, the *E. faecalis* strains in early stationary phase (grown overnight) were harvested by centrifugation (5,000 × g, 5 min, 20°C) and resuspended at a density of 10^8 bacteria/ml in culture medium without serum and antibiotics. Confluent Caco-2/TC7 cells were washed twice with fresh medium, and the bacterial suspensions were then applied at the surface of the cell monolayers.

Adhesion and cytotoxicity assays on Caco-2/TC7 cells. For adhesion, Caco-2/TC7 cells were infected with the *E. faecalis* strains for 4 h. At the end of incubation, nonadherent bacteria were removed by PBS (phosphate-buffered saline), and cells were lysed for 15 min with 0.1% Triton X-100. The lysates were diluted and plated on agar plates to determine the number of adherent/invasive bacteria.

For the cytotoxicity assay, Caco-2/TC7 cells were infected with the *E. faecalis* strains for 24 h. At the end of incubation, the cytotoxicity of the bacterial strains was estimated using two complementary methods: the lysed cells were quantified by measuring the lactate dehydrogenase (LDH) released in the cell supernatants as described by Madi et al. (23), and the viability of the cells was performed using the neutral red (NR) assay according to the procedure of Fautz et al. (10). Briefly, the cells were stained with 0.2 ml of fresh medium containing 50 μg/ml of NR for 1 h (37°C, 5% CO_2, 95% relative humidity) and were rinsed once with PBS. The NR was extracted with 1% (vol/vol) acetic acid in a 1:1 water-ethanol mixture. The absorbance of the extracted dye was spectrophotometrically measured at 540 nm.

Systemic murine infection experiments. Mouse infections were done as described by Michaux et al. (25). Briefly, bacteria were cultivated in brain heart infusion (BHI) medium with 40% heat-inactivated horse serum. Ten female BALB/c mice were infected through the tail vein with 100 μl of a PBS solution containing 10^9 bacteria/ml. After 7 days of infection, mice were euthanized by CO_2 asphyxiation. Their kidneys and their liver were removed, weighed, and homogenized in 5 ml of PBS buffer. The quantity of bacteria was determined by serial dilutions on an *Enterococcus* agar plate.

RESULTS AND DISCUSSION

Identification and mutagenesis of genes encoding PPIases in *E. faecalis*. To identify the genes encoding PPIases in *E. faecalis* V583, we searched for homologs to the characterized *S. pneumoniae* PpmA and SlrA PPIases (7, 13) using BLASTp software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (2). One *E. faecalis* V583 open reading frame (ORF) encodes a protein (EF0685) which exhibits 119/302 (39%) amino acid sequence identity and 178/302 (58%) similarity to the *S. pneumoniae* PpmA PPIase (Fig. 1A), while two genes encode SlrA homologs. Indeed, *ef1534* encodes a protein which exhibits 116/238 (48%) identity and 150/238 (63%) similarity to SlrA of *S. pneumoniae*, and a protein deduced from *ef2898* shows 94/204 (46%) identity and 131/204 (64%) similarity with this PPIase (Fig. 1B). The EF0685 and EF1534 proteins were previously predicted to be lipoproteins (31) and consequently are expected to act on folding and trafficking of extracellular proteins. EF1534 and EF2898 share a high level of homology (52% identity and 68% similarity [Fig. 1B]); however, no predicted peptide signal or transmembrane domain was found in EF2898, suggesting it could be an intracellular folding protein. The *ef0685* ORF has been annotated as a rotamase of the parvulin family in the genome of the *E. faecalis* V583 strain. It encodes a protein of 342 amino acids with a calculated molecular mass of 37.4 kDa. Both ORFs *ef1534* and *ef2898* were annotated as PPIases, encoding a 27.4-kDa protein consisting of 249 amino acids and a 196-amino-acid-long protein of 21.5 kDa, respectively.

Search for conserved domains using Pfam (http://pfam.sanger.ac.uk/search [11]) within the amino acid sequences of the 13 *E. faecalis* V583 ORFs assigned to the protein folding and stabilization role category (30) revealed the existence of a fourth PPIase. It (EF0715) contains the Pfam05697, Pfam00254, and Pfam05698 domains, which are characteristic of ribosome-associated trigger factors and consequently would be involved in the folding of newly synthesized proteins (8).

As several PPIases from Gram-positive bacteria were shown to be involved in virulence and particularly in host colonization, we investigated the role of the *E. faecalis* EF0685, EF1534, and EF2898 PPIases in stress resistance and virulence by construction of single Δ*ef0685*, Δ*ef1534*, and Δ*ef2898* mutants as described in Materials and Methods. As EF1534 and EF2898 share high similarities, and because EF1534 and EF0685 were predicted to be lipoproteins and consequently are both expected to be involved in the folding of extracellular proteins, we also constructed the Δ*ef1534* Δ*ef2898* and Δ*ef0685* Δ*ef1534* double mutants (see Materials and Methods). Since the construction of mutants with deletions of 90%, 78%, and 80% of the ORFs *ef0685*, *ef1534*, and *ef2898*, respectively, was successful, none of these genes appeared essential for *E. faecalis* growth.

Phenotypic characterization of mutants. When growth of the Δ*ef0685*, Δ*ef1534*, Δ*ef2898*, Δ*ef1534* Δ*ef2898*, and Δ*ef0685* Δ*ef1534* mutants was compared to that of the wild-type strain

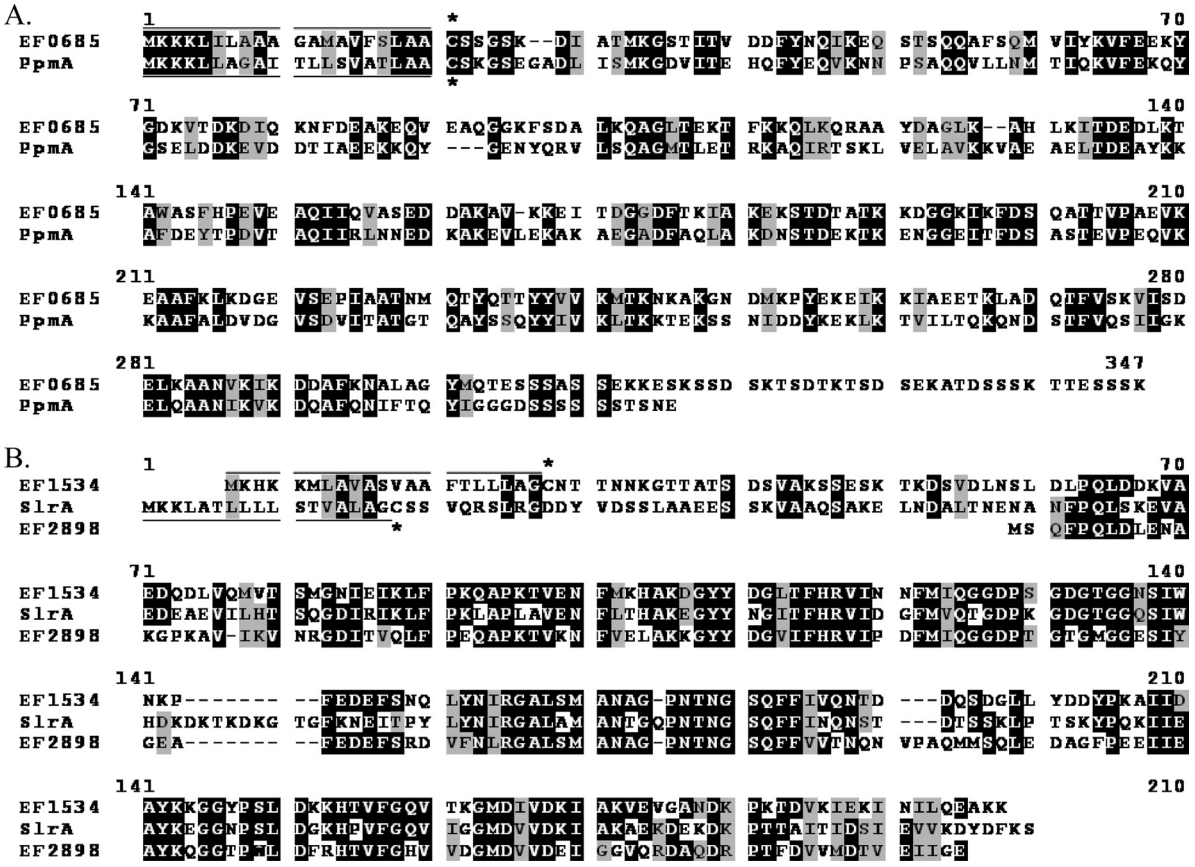

FIG 1 Alignments of EF0685, EF1534, and EF2898 with PPIases from *S. pneumoniae*. (A) *E. faecalis* EF0685 was aligned with *S. pneumoniae* PpmA, which belongs to the parvulin family. (B) *E. faecalis* EF1534 and EF2898 were aligned with *S. pneumoniae* SlrA, which belongs to the cyclophilin A family. Amino acids constituting putative peptide signals were overlined or underlined, and cysteines expected to be located in position +1 of the mature lipoproteins are indicated by asterisks. Residues identical in 2 proteins are presented on a black background, and functionally related amino acids (H, K, and R; F, Y, and W; L, I, M, and V; G and A; S and T; D and E; N and Q; and C and K) are on a gray background.

using GM17 medium with or without shaking during incubation, no significant difference was observed (data not shown).

The behavior of the wild-type and mutant strains to sodium chloride (28.5%), bile salts (0.3%), urine, ethanol (22%), and antibiotics exposure at different pH (4.0 to 9.0) or at 62°C was assayed, as indicated in Materials and Methods. The viability of Δ*ef0685* and Δ*ef1534* was affected by high sodium chloride concentration, while the complemented strains restored the wild-type phenotype (Fig. 2). Under this condition, the viability of Δ*ef0685* Δ*ef1534* was even more impaired than that of the single mutants (Fig. 2). PPIases involvement in NaCl stress was already observed in *Shewanella* sp. strain WP3 through overexpression of a gene encoding a PPIase during growth in the presence of NaCl (20). NaCl is known to generate important damages to the folding of cell surface proteins (14, 37). Consequently, EF0685 and EF1534 may be involved in the repair of damages caused by a high NaCl concentration on the surface of bacterial cells. These extracellular foldases may also play a role in the folding of ABC transporter components like the lipoprotein encoded by *ef0863*, which corresponds to the substrate binding subunit of an osmolyte transporter (OpuAC). So the NaCl sensitivity of Δ*ef0685* and Δ*ef1534* could also be explained by a reduction of osmolyte intake due to misfolding of ABC transporter components in the absence of EF0685 and EF1534.

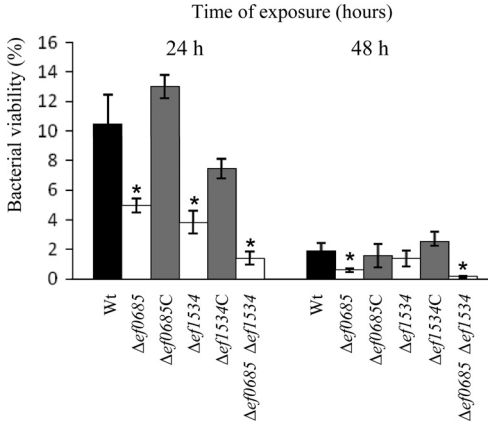

FIG 2 Effect of a high NaCl concentration on survival of wild-type and mutant strains. Bacteria grown in GM17 medium to an OD_{600} of 0.5 were harvested and resuspended in saline solution containing 28.5% of NaCl. The percent viability represents the ratio between the number of cells surviving after 24 h or 48 h and the number of cells prior to challenge. Results are those of the *E. faecalis* wild-type strain (black bars), Δ*ef0685* and Δ*ef1534* single mutants, Δ*ef0685* Δ*ef1534* double mutant (white bars), and Δ*ef0685*C and Δ*ef1534*C complemented strains (gray bars). Student's *t* test was used to determine whether results for the wild type and other strains were significantly different. *, $P < 0.05$.

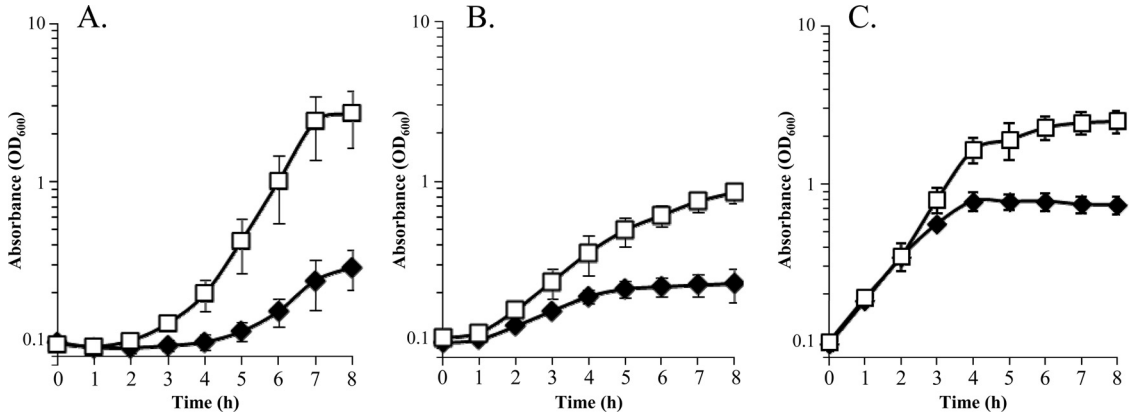

FIG 3 Effect of *ef0685* and *ef1534* inactivation on growth. Growth of the *E. faecalis* wild type (filled diamonds) and Δ*ef0685* Δ*ef1534* mutant (open squares) was determined by optical density at 600 nm (OD_{600}). (A) Growth in GM17 medium supplemented with 1.5 mM H_2O_2 under shaking; (B) growth in CCM17 MOPS medium supplemented with 0.5% glycerol under shaking; (C) growth in MCDE Caa Trp medium with 1.76 mM manganese at 37°C with shaking.

Since *E. faecalis* lipoproteins were shown to be involved in oxidative stress response (32), we analyzed growth of mutants under oxidative stress conditions. No difference with the three single mutants or with the Δ*ef1534* Δ*ef2898* double mutant was observed when they were incubated in GM17 medium supplemented with 1.5 mM H_2O_2 (data not shown). Alternatively, the Δ*ef0685* Δ*ef1534* strain appeared to have an advantage under this condition (Fig. 3A). Growth of the mutants was also evaluated in CCM17 MOPS medium supplemented with 0.5% glycerol (a condition which allows H_2O_2 production within the cytoplasm [5]) (Fig. 3B). Under this condition again, growth of the Δ*ef0685* Δ*ef1534* double mutant was less affected than that of the wild-type strain or single mutants (OD_{600} of 0.85 at 8 h for Δ*ef0685* Δ*ef1534* versus OD_{600} of 0.23 for the wild-type strain). As lipoprotein PPIases act on the conformation of extracellular proteins, we can suggest that the wild-type strain or single mutants could be targets of oxidation or could enhance the effect of oxidative stress and that their misconformation contributes to the phenotype observed here in protecting cells of oxidation.

Activity of the superoxide dismutase (SOD) was evaluated using zymographic analysis (Fig. 4) and revealed that the growth advantage for the Δ*ef0685* Δ*ef1534* double mutant under oxidative stress conditions is not due to a change in the level of the SOD activity.

Antibiograms were performed to compare antibiotic resistance levels of the mutants and the wild-type strain. Areas of growth inhibition for the single Δ*ef0685*, Δ*ef1534*, and Δ*ef2898* mutants and the Δ*ef1534* Δ*ef2898* mutant were similar to that of the wild-type strain (data not shown). However, important differences were observed when the Δ*ef0685* Δ*ef1534* double mutant was exposed to ampicillin, ofloxacin, norfloxacin, and ciprofloxacin (Table 3). Indeed, the Δ*ef0685* Δ*ef1534* mutant presented sensitivity toward ampicillin, while smaller areas of inhibition for ofloxacin, norfloxacin, and ciprofloxacin revealed resistances of the double mutant to these antibiotics from the quinolone family. Ampicillin acts by inhibiting cell wall synthesis. The inactivation of both extracellular chaperones EF0685 and EF1534 is expected to have an effect on conformation of secreted proteins, which could explain the phenotype of the Δ*ef0685* Δ*ef1534* double mutant to antibiotics interfering with the synthesis of the cell wall. The antibacterial action of quinolones is directed against the DNA replication with a second oxidative effect (9). The phenotypes of the Δ*ef0685* Δ*ef1534* double mutant in regard to ofloxacin, norfloxacin, and ciprofloxacin are probably the consequence of the difference in intake of these antibiotics due to the misconformation of extracellular proteins or could be linked to the increased ability of the corresponding mutant to grow in the presence of the oxidant.

As several membrane proteins are expected to be involved in the transport of components such as divalent transition metal ions, which are essential or in contrast could be toxic at high concentrations (21), we tested in a chemical defined medium the effect of a high manganese concentration on the growth of the wild-type strain and the mutants. No difference was observed for the Δ*ef0685*, Δ*ef1534*, and Δ*ef2898* single mutants and the Δ*ef1534*

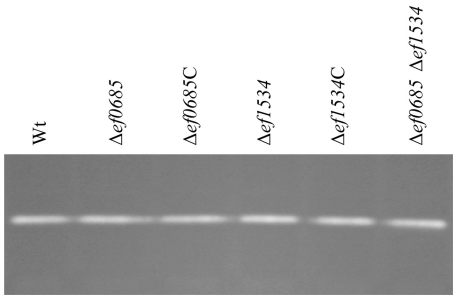

FIG 4 Activity of the superoxide dismutase enzyme detected by zymographic analysis. Proteins were extracted from the *E. faecalis* wild type, the Δ*ef0685*, Δ*ef1534*, and Δ*ef0685* Δ*ef1534* mutants, and the Δ*ef0685C* and Δ*ef1534C* complemented strains.

TABLE 3 Diameters of the inhibition areas (mm) observed with antibiogram analysis

Antibiotic	Diameter (mm) of inhibition area	
	Wild type	Δ*ef685* Δ*ef2898* mutant
Ampicillin	14.5 (±1.8)	20.5 (±0.3)
Ofloxacin	22.5 (±0.3)	13.0 (±0.1)
Norfloxacin	23.0 (±0.1)	12.5 (±0.3)
Ciprofloxacin	29.0 (±0.1)	18.0 (±0.3)

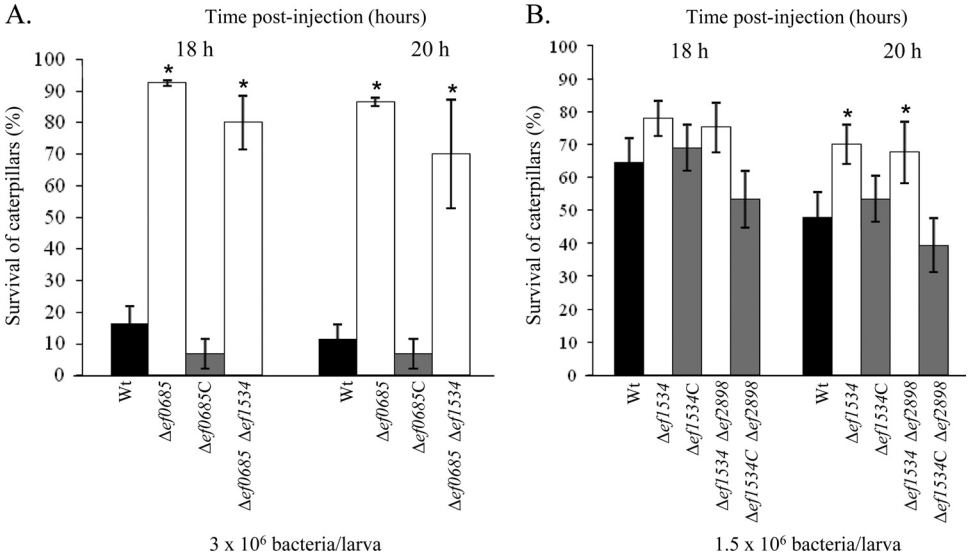

FIG 5 Effect of PPIase inactivation on *G. mellonella* survival. Percent survival of *G. mellonella* from 18 h to 20 h postinfection with the *E. faecalis* wild type, mutants, and complemented strains. (A) A total of 3×10^6 CFU counted on an agar plate of the *E. faecalis* wild type (black bars), Δ*ef0685* and Δ*ef0685* Δ*ef1534* mutant strains (white bars), and Δ*ef0685*C complemented strain (gray bar) were injected per larva. (B) A total of 1.5×10^6 CFU counted on an agar plate of the *E. faecalis* wild type (black bars), Δ*ef1534* and Δ*ef1534* Δ*ef2898* mutant strains (white bars), and Δ*ef1534*C and Δ*ef1534*C Δ*ef2898* complemented strains (gray bars) were injected per larva. Student's *t* test was used to determine whether results for the wild type and other strains were significantly different. *, $P < 0.05$.

Δ*ef2898* double mutant (data not shown). However, a clear advantage was seen for the Δ*ef0685* Δ*ef1534* double mutant for growth in the presence of manganese compared to the wild-type strain (final OD_{600} of 2.4 for the mutant versus 0.7 for the wild-type strain) (Fig. 3C). This phenotype for Δ*ef0685* Δ*ef1534* is similar to that previously observed with an *lgt* mutant (32) and probably results in both cases from a decrease in Mn^{2+} internalization. This suggests that the absence of lipidated lipoproteins in the *lgt* mutant or the absence of the EF0685 rotamase and the EF1534 PPIase is probably responsible of the misconformation of proteins involved in the transport of manganese. The better growth of the Δ*ef0685* Δ*ef1534* double mutant under oxidative stress and its capacity to growth under a high manganese concentration are phenotypes similar to those previously observed for an *lgt* mutant (32). In this *lgt* mutant, the observed corresponding phenotypes could be due to nonlipidated EF0685 and EF1534, which probably became partially inefficient and led to misconformation of secreted or surface-exposed proteins.

In the presence of H_2O_2 or with a high Mn^{2+} concentration, the Δ*ef0685* Δ*ef1534* double mutant presented a clear advantage for growth compared to the wild-type strain, while the Δ*ef0685* and Δ*ef1534* single mutants were as sensitive as the wild-type strain. Also, in the presence of a high sodium chloride concentration, the survival of the double mutant was more affected than that of single mutants, and phenotypes faced to antibiotics exposure were visible only with Δ*ef0685* Δ*ef1534*. Consequently, we can suggest that one PPIase might replace the absence of the other or if the two PPIases do not possess the same protein targets, targets of one PPIase might replace targets of the other.

Involvement of PPIase-encoding genes in *E. faecalis* fitness. As described in the introduction, PPIases were shown to be involved in the fitness and, consequently, in the virulence of Gram-positive bacteria. In order to determine the involvement of the EF0685, EF1534, and EF2898 PPIases in virulence, *Galleria mellonella* caterpillars were infected with the wild-type strain or mutants (Δ*ef0685*, Δ*ef1534*, Δ*ef2898*, Δ*ef1534* Δ*ef2898*, and Δ*ef0685* Δ*ef1534*), as described in Materials and Methods. Larvae infected with Δ*ef0685* at 3×10^6 cells/larva survived bacterial infection better, as demonstrated by the survival rate monitored at 18 h and 20 h postinfection (Fig. 5A). On the other hand, the Δ*ef1534* mutant showed a small attenuation of virulence only visible with 1.5×10^6 cells/larva (Fig. 5B), while caterpillar survival after infection with Δ*ef2898* was not significantly different from that observed with the wild-type strain (data not shown). The Δ*ef0685* Δ*ef1534* double mutant had a similar loss of virulence to Δ*ef0685* (Fig. 5A). *G. mellonella* larvae were also infected with the Δ*ef1534* Δ*ef2898* double mutant and the Δ*ef1534C* Δ*ef2898* (Fig. 5B) and Δ*ef1534* Δ*ef2898C* (data not shown) complemented strains. The Δ*ef1534* Δ*ef2898* mutant presented attenuated virulence similar to that of the Δ*ef1534* mutant, and when it was complemented with *ef1534*, bacteria recovered the wild-type phenotype (Fig. 5B, Δ*ef1534C* Δ*ef2898*). However, when the Δ*ef1534* Δ*ef2898* mutant was complemented with *ef2898*, no reduction of larval survival was observed compared to the double mutant (data not shown). Taken together, these data suggest that the extracytoplasmic foldases EF0685 and EF1534 are important for *E. faecalis* virulence but not the intracytoplasmic chaperone EF2898, and the involvement of the EF1534 PPIase was less important than that of the EF0685 rotamase. The involvement of extracellular PPIase in virulence is in accordance with results observed with the Gram-positive bacteria group A *Streptococcus* (22), *S. equi* (12), *L. monocytogenes* (1), and *S. pneumoniae* (7, 13, 28).

Further experiments were performed to determine the effect of *ef0685* and *ef1534* inactivation on adhesion to eukaryotic cells, colonization, and cytotoxicity.

To test the capacity of bacteria to adhere to eukaryotic cells, we infected Caco-2/TC7 cells with the Δ*ef0685*, Δ*ef1534*, and wild-type strains. After 4 h of incubation, the number of CFU on agar

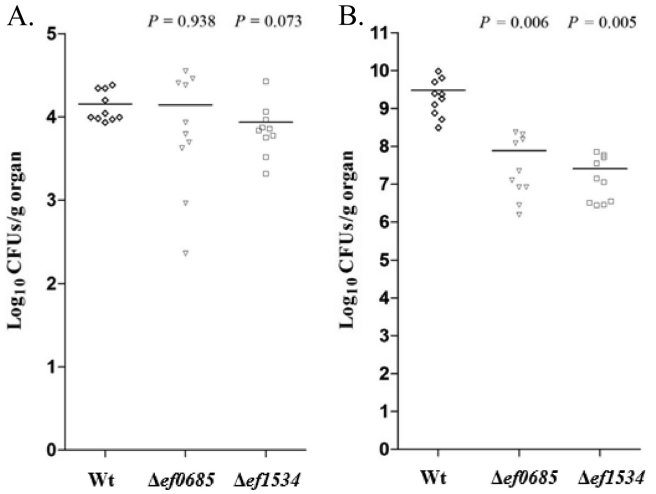

FIG 6 Effect of *ef0685* or *ef1534* inactivation on the persistence of bacteria after infection. Mice were infected with the wild-type strain and Δ*ef0685* and Δ*ef1534* mutants. After 7 days of infection, the mice were sacrificed. Organs were removed and homogenized, and bacteria on agar plates were counted. The experiment was repeated 3 times. (A) Log_{10} number of CFU per gram of liver; (B) log_{10} number of CFU per gram of kidney.

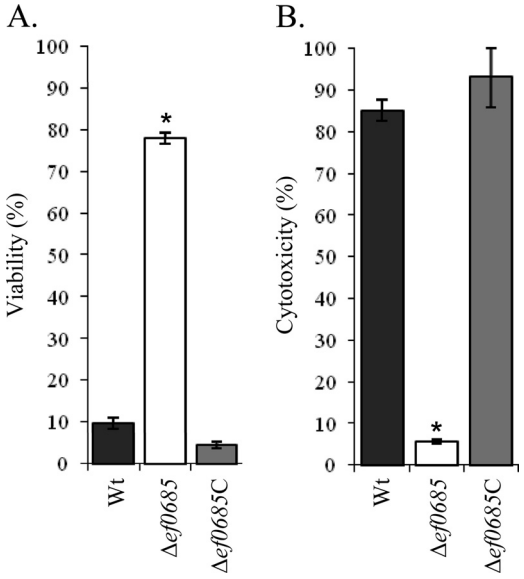

FIG 7 Effect of *ef0685* inactivation on cytotoxicity. Caco-2/T7 cells were infected with the *E. faecalis* wild type (black bars), Δ*ef0685* mutant (white bars), and Δ*ef0685C* complemented strain (gray bars). (A) Evaluation of cell survival using neutral red staining; (B) evaluation of lysed cells using the reactive LDH method. Student's *t* test was used to determine whether results for the wild type and other strains were significantly different. *, $P < 0.01$.

plates were counted. No significant difference was observed in the adhesion capacities of the Δ*ef0685* and Δ*ef1534* mutants, which succeeded in eukaryotic cells adhesion as well as the wild-type strain (data not shown).

Using systemic murine infection experiments, we followed the persistence of bacteria on two different organs: the liver and the kidneys. After 7 days postinfection, 10 female mice were sacrificed, and their organs were removed, weighed, and homogenized in PBS buffer. Then, viable bacteria were enumerated. The numbers of CFU per gram of organ determined from the liver were not significantly different between the mutants and the wild type (Fig. 6A). In contrast, the number of Δ*ef0685* or Δ*ef1534* cells in the kidneys was lower than that of the wild-type strain. Indeed, a difference of 2 log_{10} CFU per gram of organ for both mutants was observed (Fig. 6B). In conclusion, the involvement of these PPIases in colonization was tissue dependent, with the two foldases EF0685 and EF1534 having difficulties in colonizing the kidneys but not the liver. Involvement of EF0685 and EF1534 in kidney colonization could be explained by the folding of already identified surface-exposed virulence factors like Ace, the aggregation substance, or EfaA (6, 19, 34, 35) and/or other membrane proteins, including ABC transporters, which are necessary to acquire essential elements that could be in limited concentrations *in vivo*. A reduction of tissue-dependent colonization was already described for mutants affected in genes encoding SlrA or PpmA in *S. pneumoniae*. Indeed, both mutants showed a weakness to colonize mouse nasopharynx (7, 13). Interestingly, as observed here in *E. faecalis*, in *S. pneumoniae*, the PPIase which belongs to the cyclophilin family (SlrA) seemed to be less involved in colonization than the PPIase of the parvulin family (PpmA).

To continue our investigation, we tested the cytotoxicity of the Δ*ef0685* and Δ*ef1534* mutants on Caco-2/T7 cells compared to that of the wild-type-strain. The eukaryotic cells and bacteria were coincubated for 24 h. The mortality of Caco-2/T7 cells was determined by measuring the amount of released LDH enzyme in the cell supernatant, and their viability was simultaneously quantified using a neutral red staining. The deficient *ef0685* strain presented a clear loss of cytotoxicity in contrast to the wild-type strain (Fig. 7) and to the Δ*ef1534* mutant (data not shown). Indeed, the neutral red staining showed a rate of 77.9% of eukaryotic cell survival when in contact with the Δ*ef0685* mutant versus 9.5% when infected with the wild-type strain (Fig. 7A), while the LDH assay revealed 5.9% lysed eukaryotic cells after infection with the Δ*ef0685* mutant versus 85.1% for the wild-type strain (Fig. 7B). EF0685 rotamase was clearly shown to be involved in cytotoxicity. This could be attributed to a direct involvement of the EF0685 PPIase in cytotoxicity or to its involvement in folding or in secretion of toxic secreted proteins. Therefore, in addition to common roles assigned to the extracellular PPIases, EF0685 is expected to be involved in specific functions contributing to cell cytotoxicity.

Conclusion. Results presented here underline the importance of the extracellular PPIases EF0685 and EF1534 for resistance to a high NaCl concentration and to ampicillin. In the mutant deficient in both the EF0685 and EF1534 PPIases, better growth under a high manganese concentration or under oxidative stress was observed, suggesting their involvement in conformation of transporters or oxidation targets.

The Δ*ef0685* mutant displayed clear attenuated virulence traits in the *G. mellonella* model, difficulties in colonizing mouse kidneys, and loss of cytotoxicity, while the Δ*ef1534* mutant showed weak attenuated virulence traits in *G. mellonella* and difficulties in colonizing mouse kidneys. Taken together, these data showed that EF0685 and EF1534 play a role in bacterial fitness and made *E. faecalis* surface PPIases interesting targets for therapeutic treatments.

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